ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2008, 68: 347–353

Nigrostriatal projecting neurons express GDNF receptor subunit RET in adult rats

Shi-Ming He[1#], Zhen-Wei Zhao[1#], Lei Zhao[2], Xue-Lian Wang[1], Dong Jia[1], Fang Hou[1], and Guo-Dong Gao[1]*

[1] Department of Neurosurgery, Institute for functional neurosurgery P.L.A, TangDu Hospital, Fourth Military Medical University, Xi'an, P.R. China, *Email: gaoguodongmail@yahoo.com.cn; [2] Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK

Nigrostriatal neurons expressing RET protein, a receptor protein tyrosine kinase of glial cell line-derived neurotrophic factor (GDNF), were investigated in rats using retrograde neural tracing with horseradish peroxidase (HRP) combined with immunohistochemistry. HRP/RET double-labeled neurons were abundantly distributed in the substantia nigra pars compacta ipsilateral to the caudate-putamen stereotaxically injected with HRP. Almost all the HRP-labeled neurons in nigra exhibited RET-like immunoreactivity, which however constituted more than half of the RET-immunoreactive cells. Our results present morphological evidence that GDNF-RET interaction plays important roles in physiological processes of nigrostriatal neuronal circuits of mammals.

Key words: substantia nigra, RET, glial cell line-derived neurotrophic factor, horseradish peroxidase, immunohistochemistry

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF), a member of transforming growth factor beta superfamily, has been well documented for its potent survival- and neurites outgrowth-promoting ability for both normal and injured mesencephalic dopaminergic neurons in animals and latest human trials (Perez-Navarro et al. 1999, Kordower et al. 2000, Love et al. 2005, Behrstock et al. 2006). Increasing evidence has shown that GDNF plays neurotrophic roles on a wide variety of neuronal populations, e.g. the spinal or cranial motorneurons, noradrenergic neurons of the locus coeruleus, cholinergic neurons of basal forebrain as well as neurons in thalamus or hippocampus (Arenas et al. 1995, Oppenheim et al. 1995, Kordower et al. 2000, Behrstock et al. 2006). Additionally, both *in vitro* and *in vivo* studies have indicated that administration of GDNF benefits the survival of peripheral ganglia including sympathetic, sensory and enteroceptive ganglia, and even intrastriatal transplanted fetal dopaminergic grafts (Buj-bello et al. 1995, Trupp et al. 1995, 1996, Honda et al. 1999). GDNF has been regarded so far as one of the most powerful neurotrophic factors for therapeutic use in the neurodegenerative diseases including Parkinson's disease and Amyotrophic Lateral Sclerosis (Duberley et al. 1998, Walker et al. 1998, Perez-Navarro et al. 1999, Kordower et al. 2000). The diverse biological actions of GDNF are mediated *via* its two-component receptor complex, which consists of a glycosylphosphatidylinositol-anchored cell surface molecule (GFRα family member) and the RET receptor tyrosine kinase. GFRα preferably characterizes with GDNF and functions as a ligand-binding receptor, while RET works as a key receptor component involved in GDNF intracellular signaling (Trupp et al. 1996, 1997, Yu et al. 1998, Paratcha et al. 2001). So far RET protein has been found widely expressed in the central nervous system, especially in the mesencephalic dopaminergic neurons (Tsuzuki et al. 1995, Glazner et al. 1998, Walker et al. 1998, Honda et al. 1999). However, it still remains elusive whether RET protein is localized on the neostriatum-projecting neurons, which constitute the nigrostriatal dopaminegic

[#] *S.M. He and Z.W. Zhao contribute equally to this work*

Correspondence should be addressed to G.D. Gao, Email: gaoguodongmail@yahoo.com.cn

Received 24 September 2007, accepted 15 February 2008

system and degenerative cell loss of this system will cause Parkinson's disease. A double-labeling method combining HRP retrograde neural tracing with RET immunohistochemistry was performed to examine the distribution of neostriatum-projecting neurons expressing RET protein in the midbrain of rats.

METHODS

Animals, anesthesia and HRP injection

Sixteen adult male rats (R1–R16, Sprague-Dawley) weighing 250–300 g were used in the present study and randomly divided into two groups. All procedures of our experiments were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, P.R. China), and all efforts were made to minimize the number of animals used and their suffering.

The rats in group I (R1–R8) were all anaesthetized by intraperitoneal administration of sodium pentobarbital (40 mg/kg body weight). They were secured on a stereotaxic frame (Narishige, Japan), and then received stereotaxic injection of 0.1 μl of 30% HRP dissolved in 0.01 M phosphate-buffered saline (PBS; pH 7.4) in unilateral caudate-putamen according to the atlas of the rat brain (0.20 mm anterior to Bregma, 3.0 mm lateral to the midline and 5.0 mm deep from the dura mater) (Paxinos and Watson 1998). After a survival of 48 hours, they were deeply anaesthetized with an overdose of sodium pentobarbital and perfused transcardially with 100 ml of 0.01 M PBS, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The trimmed brain blocks containing caudate-putamen and mesencephalon were placed into 30% sucrose overnight at 4°C, and sliced to yield coronal sections with 30 μm thickness on a microtome. The sections were collected in 0.01 M PBS (pH 7.4), and processed for double visualization of RET protein and incorporated HRP. The group II rats (R9–R16) were processed as above except HRP injection, and performed for RET-immunohistochemistry.

Immunohistochemistry of RET protein

Immunohistochemistry was carried out to demonstrate the distribution of RET-containing structures in the rat brains as described previously (Zhou et al. 2008). Briefly, the sections were incubated sequentially with: (1) rabbit anti-RET IgG (1:500, Santa Cruz Biotechnology) in 0.01 M PBS containing 1% normal goat serum and 0.03% Triton X-100 for 48 hours at 4°C; (2) Biotinylated goat anti-rabbit IgG (Vector, 1:200 dilution) for 2 hours at room temperature; (3) ABC complex solution (Vector; 1:200 dilution) for 1 hour at room temperature. Then the sections were developed with DAB/H_2O_2 in Tris-HCl buffer (pH 7.6) for about 30 minutes. After that, they were mounted on gelatin-coated glass slides, air-dried, dehydrated, cleared and observed under an Olympus microscope (BX-60). For the control experiments in RET immunohistochemistry, the primary antibody was substituted with normal rabbit serum. The RET-like immunoreactive neurons were not found in the control experiments (data not shown).

Double-labeling of RET protein and incorporated HRP

Double-labeling experiments were performed to visualize the localization of RET protein in neostriatum-projecting neurons. For the histochemical demonstration of injected and incorporated HRP, the sections were developed with tetramethylbenzidine and sodium tungstate was used as a stabilizer (Rye et al. 1984). Then, they were treated with diaminobenzidine and cobalt chloride to enhance HRP reaction products. Subsequently, the sections were processed for RET immunohistochemistry as described above. For semi-quantification, the number of HRP single-, RET single-, HRP/RET double-labeled cells were counted in the bilateral substantial nigra pars compacta on three sets of serial sections covering the whole substantia nigra from each rat brain by a person without the knowledge of this experiment, and the data were presented as the Mean ± SD. To morphometrically measure the RET single- and HRP/RET double-labeled cells, images were captured and quantified with AxioVision Rel. 4.5 software (Zeiss). The average diameters of RET-immunoreactive or HRP/RET double-labeled neuronal somata were presented as Mean ± SD by measuring their short and long axis respectively. The nomenclature and description of brain structures were utilized according to Paxinos and Watson's Rat Brain in stereotaxic coordinates (Paxinos and Watson 1998).

RESULTS

HRP injection sites in the group I rats (R1–R8) were all exactly located within the caudate-putamen in the rostro-caudal extension from Bregma +1.4 mm to Bregma −1.2 mm. One representative injection region was shown in Fig. 1. No HRP was found spreading into the lateral ventricle or across the brain midline.

In RET-immunohistochemical stained sections, RET-like immunoreactivity was clearly shown in certain midbrain regions including substantia nigra, ventral tegmental area, red nucleus and central gray. The RET-like immunoreactivity was localized in the cell membrane, axon fibers or dendrites, and neuronal cytoplasm. In line with the previous reports (Tsuzuki et al. 1995, Trupp et al. 1997), RET-like neurons were abundantly distributed in the substantia nigra pars compacta, and most of their neuronal somata were fusiform, oval or polygonal in shape and of medium size (Fig. 2A–B). The long and short diameters of these neurons were individually measured and presented in Table I (n=106 for fusiform or oval neurons, n=110 for polygonal neurons). Some of the RET-like neurons also sparsely resided in substantia nigra pars reticularis and lateralis (data not shown).

In the double-labeled sections, both HRP-labeled and RET-immunoreactive cells were numerously found in the distinct regions of the midbrain. HRP-labeled neurons exhibited black punctuate granules in their cytoplasm and proximal dendrites. HRP/RET double-labeled neurons were exclusively distributed in the substantia nigra pars compacta with rostral-caudal extension at the ipsilateral side (Fig. 2C–F). These double-labeled neurons were characterized with the presence of black

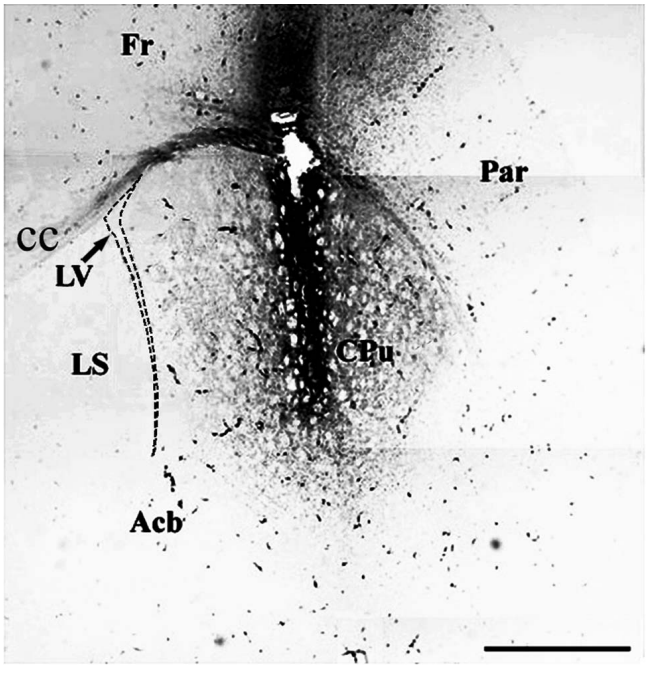

Fig. 1. One representative HRP injection region. A volume of 0.1 µl HRP (30%) was stereotaxically injected into the caudate-putamen of the rats in group I. It did not diffuse into the lateral ventrical [(LV) delineated with broken black line and indicated with black arrow], or across the midline. (Acb) accumbens nucleus; (cc) corpus callosum; (CPu) caudat-putamen; (Fr) frontal cortex; (LV) lateral ventricle; (LS) lateral septal nucleus; (Par) parietal nucleus. Scale bar is 2 mm.

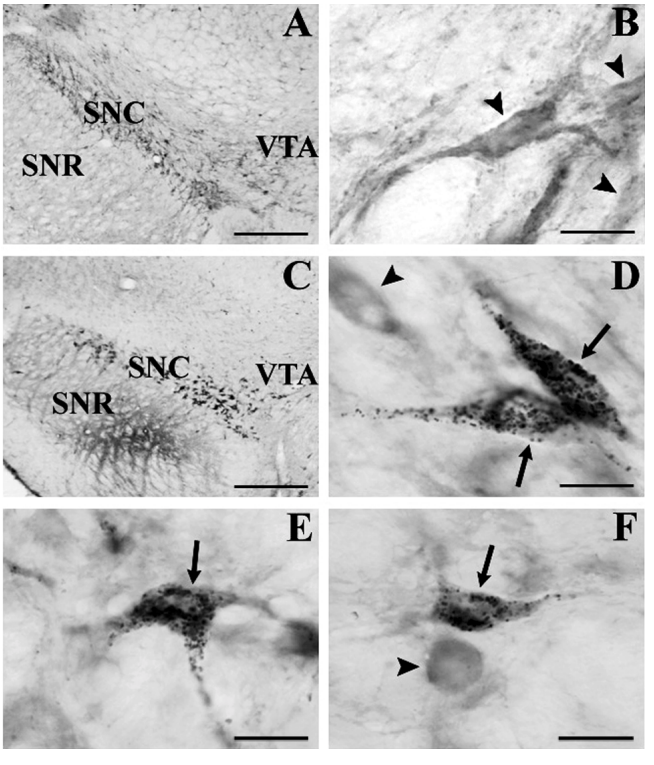

Fig. 2. RET single-labeled and HRP/RET double-labeled neurons in the substantia nigra. Single RET immunoreactive neurons were intensely located in the substantia nigra pars compacta (A), and representatively shown in (B) with higher magnification (indicated with arrowheads). HRP/RET double-labeled neurons were also robustly distributed in substantia nigra pars compacta (C). Typical double-labeled neurons were shown in (D)–(F), and indicated with arrows, while RET single-labeled neurons were indicated with arrowheads. (SNC) substantia nigra pars compacta; (SNR) substantia nigra pars reticularis; (VTA) ventral tegmental area. Scale bars are 300 µm in (A) and (C), and 15 µm in (B), (D), (E), and (F).

Table I

Morphometric measurement of RET single- or HRP/RET double-labeled neurons in the substantia nigra pars compacta of rats receiving HRP injection

	Fusiform (*n*=106)		Oval (*n*=106)		Polygonal (*n*=110)	
	long (μm)	short (μm)	long (μm)	short (μm)	long (μm)	short (μm)
RET single-labeled	27.59 ± 3.83	13.69 ± 2.91	16.83 ± 3.65	13.17 ± 2.77	17.75 ± 4.24	13.61 ± 2.88
HRP/RET double-labeled	28.93 ± 3.15	12.64 ± 2.17	15.82 ± 3.03	14.94 ± 2.05	18.77 ± 3.74	15.76 ± 2.76

Long and short represent the long or short axis of measured cells, respectively. Data were presented as Mean ± SD.

punctuate granules (HRP-reactive products) within the brown-colored cytoplasm (RET-like immunoreactivity) throughout neuronal somata or processes. Most of the HRP/RET double-labeled neurons were medium-sized and displayed similar morphology as the RET single-labeled neurons, which can be further divided into fusiform, oval or polygonal subgroups. Again, the long and short axis of these neurons were individually measured and shown in Table I (*n*=106 for fusiform or oval neurons, and *n*=110 for polygonal neurons). HRP single-, RET single-, and HRP/RET double-labeled neurons were counted in substantia nigra pars compacta for semi-quantification. Most HRP-labeled neurons exhibited RET-like immunoreactivity. However, although HRP/RET double-labeled neurons constituted more than half of the total RET-like cells (Table II), it should be noted that there were still some RET single-labeled cells in these regions. Because the incorporated HRP was further evidenced by the method of cobalt chloride enhancement (Rye et al. 1984), it should be taken into

Table II

The number of HRP single-, RET single-, and HRP/RET double-labeled neurons in the substantia nigra pars compacta of rats

	HRP		RET		HRP/RET	
	ipsi	contra	ipsi	contra	ipsi	contra
R1	42 ± 11	0	1041 ± 74	3878 ± 202	3086 ± 67	0
R2	79 ± 12	0	1748 ± 96	3858 ± 224	2817 ± 50	0
R3	90 ± 11	0	1733 ± 123	3197 ± 135	1759 ± 193	0
R4	133 ± 23	0	1881 ± 169	2238 ± 187	2156 ± 230	0
R5	131 ± 23	0	1671 ± 209	2055 ± 234	1947 ± 111	0
R6	86 ± 6	0	1684 ± 105	2065 ± 88	1866 ± 109	0
R7	106 ± 17	0	1466 ± 86	2247 ± 232	1828 ± 62	0
R8	138 ± 17	0	1829 ± 88	2273 ± 226	2039 ± 151	0

HRP single-, RET single- and HRP/RET double-labeled neurons in ipsilateral (ipsi) and contralateral (contra) substantia nigra were counted on three sets of serial sections covering the whole substantia nigra in each rat, and the results were presented as Mean ± SD. R1–R8 represent the eight rats that received HRP injection.

consideration that some mild or moderate HRP-containing neurons might become undetectable due to its decreased sensitivity. Therefore, the ratio of the double-labeled neurons might be underestimated.

DISCUSSION

Distribution of *c-Ret* mRNA or protein in the mesencephalic dopamine neurons has been well examined in previous *in situ* hybridization and immunohistochemical observations (Tsuzuki et al. 1995, Trupp et al. 1997, Glazner et al. 1998). In agreement with these reports, our results further demonstrate that a high percentage of the neostriatum-projecting neurons express the GDNF receptor subunit tyrosine kinase RET in the rat brain. However, there are still many RET single-labeled cells distributed in substantia nigra pars compacta. One possible explanation is that not all the HRP reactive products were well presented due to the limitation of the staining method we used. Alternatively, the existence of RET single-labeled cells indicates that RET protein may be implicated in multiple signaling pathways in the substantia nigra in adult rats. The HRP/RET double-labeled neurons present similar morphology to the RET single-labeled neurons suggests that they may be morphologically homogenous but exert distinct functions.

It is well known that GDNF can prevent the neurons exposed to neurotoxins from degeneration, promote neuronal axon sprouting and increase tyrosine hydroxylase level in substantia nigra dopamine neurons through its receptor complex (Perez-Navarro et al. 1999, Kordower et al. 2000). Three mammalian homologues of GDNF have been so far identified, and RET protein acts as their common signaling receptor related to different members (GFRα-1 to -4) of the GFRα family (Klein et al. 1997, Baloh et al. 1998, Kjaer et al. 2003, Sariola et al. 2003). Mice with null mutations of RET exhibit similar phenotypic defects as the GDNF knockout mice, including severe dysgenesis and lack of enteric neurons, indicating that RET is not only involved in GDNF signaling in adult CNS but a key component in the developing peripheral nervous system (Trupp et al. 1995, Sanchez et al. 1996). RET protein and GFRα have distinct but partially overlapping distribution patterns in the mammalian brain, which implicates that they may function independently or synergically (Trupp et al. 1997). In the dopaminergic neurons of midbrain, RET tyrosine kinase co-localizes with GFRα (Trupp et al. 1997, Yu et al. 1998), which in turn makes the protein more efficiently activated by GDNF (Trupp et al. 1996, 1997, Yu et al. 1998, Paratcha et al. 2001).

Interestingly, expression of the RET protein can be transiently up-regulated in the hippocampus and spinal motorneurons after ischemia (Sakurai et al. 1999, Miyazaki et al. 2002), indicating that trophic circuits of GDNF may be modulated dynamically at the level of the receptors. In patients with Parkinson's disease, RET protein can still be found in surviving dopaminergic neurons of substantia nigra (Walker et al. 1998, Kordower et al. 2000). It has been shown that constitutive RET activity in mice is sufficient to increase the number of dopaminergic neurons and leads to profound elevation of brain dopamine concentration (Mijatovic et al. 2007). Therefore, further understanding on how trophic factors respond to the ongoing degenerative process and how RET protein actively shapes the brain dopaminergic system may be important to restore the damaged neuronal circuit and to develop effective treatment strategies.

CONCLUSION

We have now demonstrated that if not all, most of the nigrostriatal projecting neurons express GDNF receptor RET protein, which provides further morphological evidence for the neurotrophic functions of GDNF-RET signaling in the dopaminergic neuronal network. This finding may be of importance for the understanding and treatment of Parkinson's disease.

ACKNOWLEDGEMENTS

We thank Dr. Liang-Wei Chen (Institute of Neurosciences, Fourth Military Medical University, P.R. China) for his critical reading the manuscript and technical assistant. This study is supported by the ChuangXin grant (CX01f004), FMMU, P.R. China.

REFERENCES

Arenas E, Trupp M, Akerud P, Ibanez CF (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron 15: 1465–1473.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EM, Milbrandt J (1998) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRa3-RET receptor complex. Neuron 21: 1291–1302.

Behrstock S, Ebert A, McHugh J, Vosberg S, Moore J, Schneider B, Capowski E, Hei D, Kordower J, Aebischer P, Svendsen CN (2006) Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. Gene Ther 13: 379–388.

Buj-bello A, Buchman VL, Horton A, Rosenthal A, Davies AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron 15: 821–828.

Duberley RM, Johnson IP, Martin JE, Anand P (1998) RET-like immunostaining of spinal motoneurons in amyotrophic lateral sclerosis. Brain Res 789: 351–354.

Glazner GW, Mu X, Springer JE (1998) Localization of glial cell line-derived neurotrophic factor receptor alpha and c-ret mRNA in rat central nervous system. J Comp Neurol 391: 42–49.

Honda T, Takahashi M, Sugiura Y (1999) Co-localization of the glial cell-line derived neurotrophic factor and its functional receptor c-RET in a subpopulation of rat dorsal root ganglion neurons. Neurosci Lett 275: 45–48.

Kjaer S, Ibanez CF (2003) Identification of a surface for binding to the GDNF-GFR alpha 1 complex in the first cadherin-like domain of RET. J Biol Chem 278: 47898–47904.

Klein RD, Sherman D, Ho WH, Stone D, Bennett GL, Moffat B, Vandlen R, Simmons L, Gu Q, Hongo JA, Devaux B, Poulsen K, Armanini M, Nozaki C, Asai N, Goddard A, Phillips H, Henderson CE, Takahashi M, Rosenthal A (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387: 717–721.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Déglon N, Aebischer P (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290: 767–773.

Love S, Plaha P, Patel NK, Hotton GR, Brooks DJ, Gill SS (2005) Glial cell line-derived neurotrophic factor induces neuronal sprouting in human brain. Nat Med 11: 703–704.

Mijatovic J, Airavaara M, Planken A, Auvinen P, Raasmaja A, Piepponen TP, Costantini F, Ahtee L, Saarma M (2007) Constitutive Ret activity in knock-in multiple endocrine neoplasia type B mice induces profound elevation of brain dopamine concentration via enhanced synthesis and increases the number of TH-positive cells in the substantia nigra. J Neurosci 27: 4799–4809.

Miyazaki H, Nagashima K, Okuma Y, Nomura Y (2002) Expression of Ret receptor tyrosine kinase after transient forebrain ischemia is modulated by glial cell line-derived neurotrophic factor in rat hippocampus. Neurosci Lett 318: 1–4.

Oppenheim RW, Houenou LJ, Johnson JE, Lin LF, Li L, Lo AC, Newsome AL, Prevette DM, Wang S (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 373: 344–346.

Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibáñez CF (2001) Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron 29: 171–184.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. Academic Press, San Diego, CA.

Perez-Navarro E, Arenas E, Marco S, Alberch J (1999) Intrastriatal grafting of a GDNF-producing cell line protects striatonigral neurons from quinolinic acid excitotoxicity in vivo. Eur J Neurosci 11: 241–249.

Rye DB, Saper CB, Wainer BH (1984) Stabilization of the tetramethylbenzidine (TMB) reaction product: application for retrograde and anterograde tracing, and combination with Immunohistochemistry. J Histochem Cytochem 32: 1145–1153.

Sakurai M, Hayashi T, Abe K, Yaginuma G, Meguro T, Itoyama Y, Tabayashi K (1999) Induction of glial cell line-derived neurotrophic factor and c-ret porto-oncogene-like immunoreactivity in rabbit spinal cord after transient ischemia. Neurosci Lett 276: 123–126.

Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382: 70–73.

Sariola H, Saarma M (2003) Novel functions and signalling pathways for GDNF. J Cell Sci 116: 3855–3862.

Trupp M, Rydén M, Jörnvall H, Funakoshi H, Timmusk T, Arenas E, Ibáñez CF (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130: 137–148.

Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, Kilkenny C, Salazar-Grueso E, Pachnis V, Arumäe U (1996) Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature 381: 785–789.

Trupp M, Belluardo N, Funakoshi H, Ibanez CF (1997) Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. J Neurosci 17: 3554–3567.

Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, Asai J (1995) Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene 10: 191–198.

Walker DG, Beach TG, Xu R, Lile J, Beck KD, McGeer EG, McGeer PL (1998) Expression of the proto-oncogene Ret, a component of the GDNF receptor complex, persists in human substantia nigra neurons in Parkinson's disease. Brain Res 792: 207–217.

Yu T, Scully S, Yu Y, Fox GM, Jing S, Zhou R (1998) Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. J Neurosci 18: 4684–4696.

Zhou H, Tang T, Guo C, Zhang H, Zhong J, Zheng J, Luo J, Lin Y, Liu W, Luo W, Yang Q (2008) Expression of Angiopoietin-1 and the receptor Tie-2 mRNA in rat brains following intracerebral hemorrhage. Acta Neurobiol Exp (Wars) 68: 147–154.